Specific Early-G_1 Blocks Accompanied with Stringent Response in *Saccharomyces cerevisiae* Lead to Growth Arrest in Resting State Similar to the G_0 of Higher Eucaryotes

HIDETOSHI IIDA AND ICHIRO YAHARA
The Tokyo Metropolitan Institute of Medical Science, Tokyo 113, Japan

ABSTRACT Growth arrests of *Saccharomyces cerevisiae* cells in early G_1 phase brought by various means were classified into two types according to the mode of growth recovery after release of the restraints against growth. The first type, including arrests caused by *cdc25*, *cdc33*, *cdc35*, and *ils1* mutations at the nonpermissive temperature and also by sulfur starvation, showed a subsequent delay in the onset of budding when shifted back to permissive conditions. The length of the delay was positively correlated with the time that cells had been arrested. The second type, including those caused by *cdc28* and *cdc24* mutations and by α factor, did not affect the mode of growth recovery after the shift to permissive conditions irrespective of the time that cell proliferation had been restricted. Growth arrests of the first type seem to allow yeast cells to enter a resting state equivalent to the G_0 state of higher eucaryotes because features of the G_0 shown with lymphocytes and other cultured cells including unusually long delay before the growth recovery (L. H. Augenlicht and R. Baserga, 1974, *Exp. Cell Res.*, 89:255–262; and Kumagai, J., H. Akiyama, S. Iwashita, H. Iida, and I. Yahara, 1981, *J. Immunol.*, 126:1249–1254) appeared to be associated with this type. We have noted that arrests of the first type were always accompanied with a stringent response of macromolecular synthesis and its partial release by cycloheximide. Mapping of arrest points along the path of the cell cycle by the reciprocal shift experiment suggested that arrest points in G_1 that led to the G_0-like arrest precede or are near the step sensitive to α-factor.

It is known that only a minor population of cells are proliferating in most tissues of adult animals and other higher eucaryotes and the remainders are resting in the G_0 state, a specific state different from the proliferating state in the cell cycle (41). A specific mechanism seems to operate, when required, in normal tissues so that cells cease from growing and enter G_0 where they often undergo differentiation (7, 21). The cellular transition from the proliferating state to the resting state has been revealed with experimental systems in vitro to have the same features as that observed in vivo (19, 22, 39). Using cultured mammalian cells, Pardee (28) has shown that growing cells reached the same quiescent state by a variety of means and re-entered the cell cycle at the same point in G_1 when the restrictions for growth were released.

To investigate further the specific point(s) in the cell cycle at which cells enter G_0, we used the budding yeast, *Saccharomyces cerevisiae*, because a series of cell division cycle (*cdc*) mutants have been isolated and the sequence of cellular events along the cell cycle directed by *CDC* genes have been analyzed to a considerable degree (30). In addition, there are at least three methods, starvation for an essential nutrient such as sulfur (11, 14), incubation of haploid yeast cells with the mating factor produced by cells of the opposite mating type, which brought about arrest of the cell division cycle in early steps of the G_1 phase (2), and incubation of temperature-sensitive (*ts*) aminoacyl-tRNA synthetase mutant at the nonpermissive temperature (44). First of all, we sought to demonstrate that yeast cells entered a resting state similar to G_0 in higher eucaryotes when the growth was appropriately arrested in G_1.

A distinctive property of cultured cells existing in G_0 may be an unusually long delay between the shift of the culture

THE JOURNAL OF CELL BIOLOGY · VOLUME 98 APRIL 1984 1185–1193

from nonpermissive to permissive conditions for growth and the initiation of DNA synthesis (1, 25). Recently, we observed that lymphocytes that had divided recently responded quickly to a stimulus, but lost this ability and became cells that responded slowly when incubated in the absence of the stimulus (19). We supposed that this alteration during the arrest in the mode of the restimulation would be a result of the entrance into G_0.

Arrests of yeast cells in specific steps between the cell division and the initiation of DNA synthesis may be classified into two types according to the mode of restimulation after arrested cells are transferred to permissive conditions for growth. First, there would be specific arrests that cause a subsequent delay in the onset of DNA synthesis upon the shift back to proliferating conditions. The length of the delay is positively correlated with the time that cells have been arrested. Second, there would be another type of specific arrests that do not affect the mode of restimulation irrespective of the time that cell proliferation has been restricted. We refer to the first type as G_0 arrest because of the similarity of the arrested yeast cells to G_0 fibroblasts and other cultured cells (1, 25) and G_0 lymphocytes (19). In addition, we have noted that G_1 arrests leading to G_0 state were always accompanied with a stringent response of macromolecular synthesis.

Relative positions of the arrest points that led to G_0 arrest or durable G_1 arrest were then mapped along the path of the cell cycle by the reciprocal shift experiments between permissive and nonpermissive conditions for growth (15, 16). The results obtained with an *ils1* mutant and those previously reported (3) suggest that arrest points leading to G_0 precede those resulting in non-G_0 arrests. This conclusion must await further evidence, however, because the results obtained with three other G_0 arrest mutants, *cdc25*, *cdc33*, and *cdc35* did not unequivocally support it.

MATERIALS AND METHODS

Strains: Genotypes and sources of the haploid strains of *S. cerevisiae* used in this study are listed in Table I.

Media: A synthetic liquid medium, SD20 (6), composed of 6.7 g/l of Bacto Yeast Nitrogen Base (Difco Laboratories, Detroit, MI) and 20 g/l of dextrose (Difco Laboratories) was used for the preparation of α-factor. The composition of SD10-S was essentially the same as Bacto Yeast Nitrogen Base (Difco Laboratories), except that all the salts containing sulfates were substituted by those containing chlorides, methionine was omitted, and asparagine (1.5 g/l) and dextrose (10 g/l) were added. SD10 was made by substituting ammonium sulfate for ammonium chloride in SD10-S. Another synthetic liquid medium (9), abbreviated hereafter as SYE, was also used with a slight modification that the concentration of uracil was 2.2 mg/l. To cultivate strains 182-6-3 and BR214-4a, we used SYE supplemented with 20 mg/l arginine, 20 mg/l tryptophan, and 200 mg/l threonine and SYE supplemented with 20 mg/l arginine and 20 mg/l tryptophan, respectively.

α-Factor: From a culture of X2180-1B, α-factor was partially purified according to the procedure described by Duntze et al. (6). The biological activity of α-factor was determined by the agar diffusion assay method of Duntze et al. (6).

Determination of Buds: Aliquots (0.1 ml) were pipetted from cultures at the indicated times and were added to the equal volume of 7.4% formaldehyde solution in PBS. The mixtures were then sonicated with a Branson Sonifier, Cell Disruptor 200 (Branson Sonic Power Co., Banbury, CT) at a power unit of 2 for 5 s to disperse cell clumps. The number of total cells and that of budded cells were determined microscopically on the fixed and briefly sonicated samples. Small buds were defined as those smaller than a half in diameter of the mother cell grown under permissive conditions. At least 300 cells were examined to determine the population of budded cells.

Sulfur Starvation: X2180-1A cells were grown in 10 ml of SD10 medium at 30°C to the cell density of 2×10^7/ml. The cells were harvested, washed three times with SD10-S medium, and suspended in 10 ml of the same medium. After incubation at 30°C for the indicated periods, the cells were transferred to 10 ml of SD10 medium. Bud emergence was determined as a function of time after the shift to SD10 medium.

TABLE I
List of Strains

Strain	Genotype	Source
X2180-1A	*MATa SUC2 mal gal2 CUP1*	YGSC*‡
X2180-1B	*MATα SUC2 mal gal2 CUP1*	YGSC‡
A364A	*MATa ade1 ade2 ural his7 lys2 tyr1 gall*	YGSC
314	*MATa cdc4-1 ade1 ade2 ural his7 lys2 tyr1 gall*	YGSC
182-6-3	*MATa cdc24-1 ural tyr1 arg4 thr4 ade his trp gal*	YGSC
321	*MATa cdc25-1 ade1 ade2 ural his7 lys2 tyr1 gall*	YGSC
185-3-4	*MATa cdc28-1 ade1 ade2 ural his7 lys2 tyr1 gall*	YGSC
E17	*MATa cdc33-1 ade1 ade2 ural his7 lys2 tyr1*	L. H. Hartwell
BR214-4a	*MATa cdc35 ural his7 tyr1 trp1 arg4 ade(1 or 2) iso(1 ?)*	L. H. Hartwell
341	*MATa ils1-1 ade1 ade2 ural his7 lys2 tyr1 gall*	YGSC

* YGSC, Yeast Genetic Stock Center, University of California, Berkeley, California.
‡ These two strains were provided by Y. Ohsumi (University of Tokyo, Tokyo, Japan).

Temperature Block: Cells of *ts*-mutants including all the *cdc* and *ils1* mutants were grown in SYE medium at the permissive temperature, 23°C. The generation time of the cells was between 140 to 170 min, but it was 270 min for E17 (*cdc33*). Exponentially growing cells (1.5×10^6/ml) were incubated at the nonpermissive temperature, 38°C for *cdc28*, 36°C for other *cdc* mutants and 32°C for *ils1* mutant, for the indicated periods. The temperature block was then released by transferring the cells to 23°C. Budding and DNA synthesis were monitored after the shift down of the temperature.

α-Factor Block: Cells of strain 321 were grown in SYE medium at 23°C to 1.5×10^6/ml, after which α-factor was added to give a final concentration of 25 units/ml. The cultures were incubated for various times, after which the block was released by washing the cells with SYE medium in Nalgene filter units (pore size 0.45 μm, Nalge Sybron Co., Rochester, NY). The cells were resuspended in fresh SYE medium and incubated at 23°C. Bud emergence and DNA synthesis were then determined.

Reciprocal Shift Experiment: The methods described by Hereford and Hartwell (15) were generally followed.

Cell Volume: The volume of individual cells was determined with a Coulter counter model ZB equipped with a pulse-height channelyzer and XY-plotter (Coulter Electronics, Inc., Hialeah, FL) after sonicating the cultures to disperse cell clumps.

Determination of Macromolecular Synthesis: The synthesis of RNA and protein was determined by incorporation of appropriate radiolabeled precursors into 5% trichloroacetic acid-insoluble materials. The precipitates were collected on GH/C filters (Whatman Inc., Clifton, NY), washed five times with 5 ml of 5% trichloroacetic acid, and then rinsed twice with 5 ml of methanol. The radioactivity retained on each filter was determined in 10 ml of Toluen-Omnifluor (New England Nuclear, Boston, MA) in a liquid scintillation counter.

rRNA Synthesis: Cultures (20 ml) in exponentially growing phase were incubated for several generation-times in the permissive condition in SD10 medium containing 0.05 μCi/ml [2-^{14}C]uracil (0.25 Ci/mol, Amersham Japan, Tokyo, Japan) for strain X2180-1A or in SYE medium containing 0.05 μCi/ml [2-^{14}C]uracil (2.4 Ci/mol) for other stains. The cultures were divided into four equal parts, two of which were then incubated for 30 min with 3.8 μCi/ml [6-^{3}H]uracil (19 Ci/mol, Amersham Japan) for X2180-1A or with 11.4 μCi/ml [6-^{3}H]uracil (554 Ci/mol) for other strains in the permissive condition in the presence or absence of 100 μg/ml cycloheximide. The other two cultures were kept for another 2 h in the nonpermissive condition and then incubated for additional 30 min with the same concentration of [6-^{3}H]uracil as described above in the presence or absence of 100 μg/ml cycloheximide. The labeled cells were washed with 5 ml of cold 0.1 M NaCl–0.01 M Tris-HCl buffer (pH 7.4, at 25°C), resuspended in 0.25 ml of the same buffer containing 0.01 M EDTA

and 0.2% (wt/vol) SDS (NETS) (24), and lyzed by voltexing with 0.3 g of 0.5-mm glass beads for 3 × 30 s. RNA was extracted from the lysates according to the method described by Udem and Warner (43). Ethanol-precipitated RNA was finally dissolved in 50 μl TPE (0.036 M Tris, 0.03 M NaH_2PO_4, 0.001 M EDTA, 0.2% [wt/vol] SDS, pH 8.0) (24) containing 6% (wt/vol) sucrose and 0.005% (wt/vol) bromophenol blue, and electrophoresed in 2.5% polyacrylamide gel according to the procedure of Udem and Warner (43). The gels were sliced, after which each slice was dissolved in 0.5 ml of 90% NCS (Amersham Japan) and the radioactivity associated with the slice was determined in 14.5 ml toluene-based scintillation fluid (New England Nuclear). The total radioactivity associated with 18S, 25S, and 35S precursor rRNAs was obtained, after which the rate of rRNA synthesis was calculated as Σ [^{3}H] dpm/Σ [^{14}C] dpm.

RESULTS

Lag of Growth Recovery from G_1 Arrest

Yeast cells can be arrested in G_1 of the cell cycle by various methods; nutrient starvation (11, 14), incubation of *ts* mutants at the nonpermissive temperature (11, 14, 44), or incubation of haploid cells with the mating factor produced by the opposite mating type cells (2). G_1 arrests caused by various methods were compared for the relationship between duration of arrest and kinetic mode of growth recovery from the arrest.

SULFUR STARVATION: X2180-1A cells were incubated in sulfur-free (SD10-S) medium at 30°C up to 64 h. The proportion of budded cells decreased and reached a minimum (~5%) in 16 h. This datum indicates that most of the cells in the culture entered G_1 during this period. The starved cells were transferred to complete (SD10) medium at the indicated times. The proportion of budded cells was determined as a function of time after the shift to SD10 medium (Fig. 1*A*). Bud emergence has been shown to occur concomitantly with the initiation of DNA synthesis (37, and our unpublished observations). The results showed that time lags between the shift and the onset of budding were 70, 110, and 150 min for cells starved for 16, 40, and 64 h, respectively. The increasing rate and the maximum level of the proportion of budded cells did not significantly differ among the three starved cultures.

ts cdc MUTANTS: Mutants 321, E17, BR214-4a, and 185-3-4 have *ts*-defects in genes *CDC25*, *CDC33*, *CDC35*, and *CDC28*, respectively. The function of these genes is necessary for the start of the cell division cycle (11, 14, 30).

Three cultures were made up for each of the above strains, and incubated at nonpermissive temperature, 38°C for *cdc28* and 36°C for other *cdc* mutants, for different times. The proportion of budded cells decreased during the incubation of nonpermissive temperature, after which the cultures were then shifted to permissive temperature, 23°C. Bud emergence was determined as a function of time after the shift down of the temperature (Fig. 1, *B–E*). For a mutant 185-3-4 (*cdc28*), only cells with small buds were used as an indication of the growth recovery because cells with large buds were indistinguishable from those with "shmoo" shape, an abnormal morphology induced in this mutant at 38°C (10). The duration of arrest induced with *cdc25*, *cdc33*, and *cdc35* mutants affected the following growth recovery at permissive temperature in the manner similar to that observed for sulfur starvation (Fig. 1, *A–D*). By contrast, kinetics of emergence of newly formed buds did not differ among the cultures of *cdc28* mutant that had been incubated at 38°C for different times (Fig. 1*E*).

Other *ts cdc* mutants examined were 182-6-3 (*cdc24*) and 314 (*cdc4*), which have defects in bud emergence and initiation of DNA synthesis, respectively (11, 14, 30). The kinetics of growth recovery was not influenced by the duration of arrest at 36°C in *cdc24* mutant (Fig. 1*F*). Cells of *cdc4* mutant rapidly lost the viability during the arrest probably because of unbalanced growth at 36°C. Surviving *cdc4* cells frequently

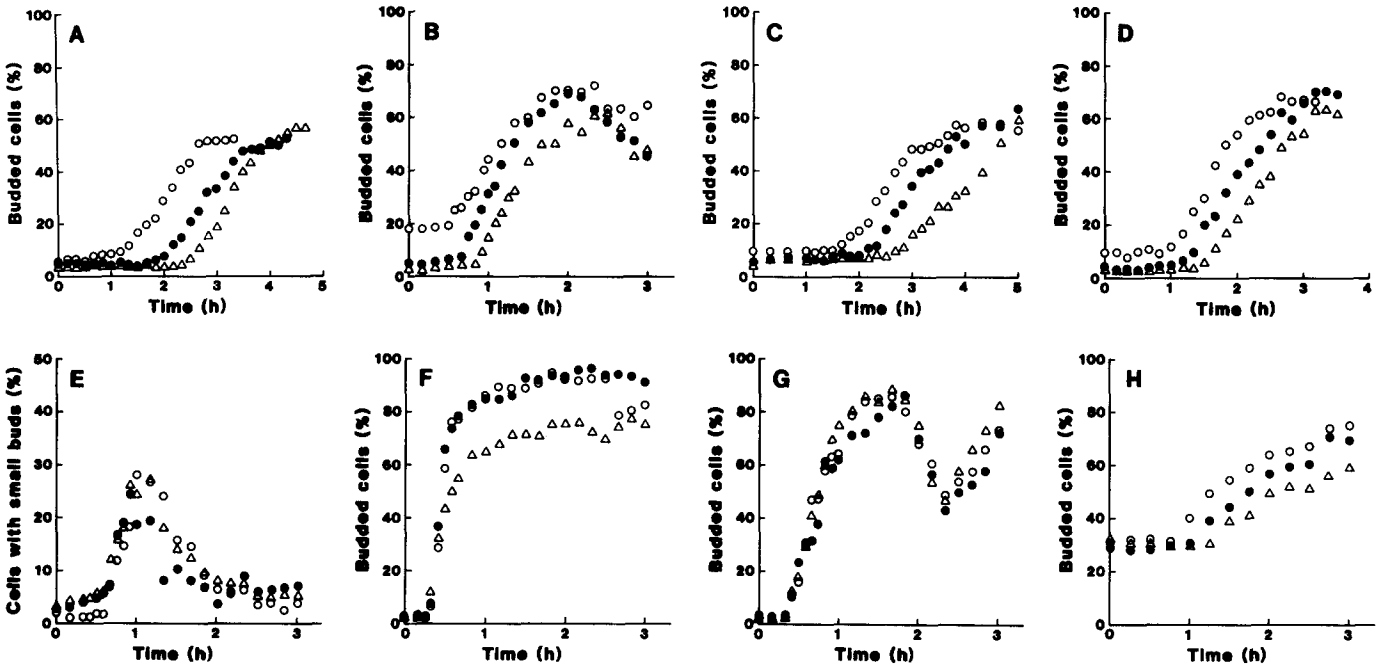

FIGURE 1 Delay in growth recovery after release from different G_1 arrests. Kinetics of bud emergence was determined after release from G_1 arrests caused by different methods: (*A*) Sulfur starvation for 16 (○), 40 (●), and 64 (△) h. (*B–H*) Temperature block. (*B*) *cdc25*, (*D*) *cdc35*, (*E*) *cdc28*, and (*F*) *cdc24* incubated at 36°C (38°C for *cdc28*) for 3.5 (○), 6 (●), and 8.5 (△) h. (*C*) *cdc33* at 36°C for 8 (○), 13 (●), and 18 (△) h. Doubling time of this strain was ~1.8 times longer than that of other *ts*-mutants. Incubation periods of this strain at the nonpermissive temperature were, therefore, elongated. (*G*) α-factor block at 23°C for 3.5 (○), 6 (●), and 8.5 (△) h. (*H*) *ils1* at 32°C for 3.5 (○), 6 (●), and 8.5 (△) h.

formed multiple buds per cell upon the growth recovery at 23°C. For these reasons, a kinetic study of the growth recovery could not be performed with this mutant.

α-FACTOR BLOCKADE: Cells of strain 321 (*cdc25-1*) (*a* mating type) were incubated with α-factor (25 units/ml) for various times, and washed to remove the mating factor. The cells were then allowed to initiate growth in medium free of α-factor. The results showed that neither a lag of 20 min before the beginning of bud emergence nor the rate of increase in number of budded cells were changed by varying the arrest period with α-factor (Fig. 1 *G*). We tested and excluded the trivial possibility that the above results might be attributed to a partial depletion of α-factor during the arrest. The results were not changed at all, however, when additional α-factor (25 units/ml) was supplied again 2.5 h before the termination of the arrest (data not shown).

Ts-AMINOACYL-tRNA SYNTHETASE MUTANT: An incubation of 341 cells (*ils1*) (12, 26) at 36°C blocked both the initiation of DNA synthesis and cell division (data not shown). An incubation at the intermediate nonpermissive temperature, 32°C blocked only the former resulting in G_1 arrest, however. As seen in Fig. 1 *H*, a delay in growth recovery after release from the temperature block was affected by the duration of the G_1 arrest. Another *ts*-aminoacyl-tRNA synthetase haploid mutant, H19-3-4 (*mes1*), which is defective in methionyl-tRNA synthetase, does not converge to single unbudded cells at 36°C (unpublished observations), while corresponding diploid cells arrest in G_1 at 36°C (44). Cytokinesis of H19-3-4 cells appeared to be inhibited at 36°C (unpublished observations). This strain could not be used in this study, therefore.

The above results indicate that G_1 arrests can be classified into two types. The first type was shown to influence the kinetics of growth recovery depending upon the duration of arrest. The second type did not alter the mode of restimulation irrespective of the duration of arrest.

Other Criteria for G_0

We tested whether or not the cell volume decreased during G_1 arrests produced by various methods. Changes in the cell volume during incubation of two *cdc* mutants, *cdc25* and *cdc28*, are shown in Fig. 2. The cell volume of *cdc28* cells increased during the incubation, whereas that of *cdc25* increased to a lesser extent. Values of the cell volume at the peak position of the distribution curve were not changed for *cdc25* cells, suggesting that the majority of these cells maintained the initial cell volume during the incubation at nonpermissive temperature. When the growth of *cdc25* cells were arrested by α-factor, the cell volume significantly increased (Table II).

The mean cell volume increased at most twofold when *cdc25*, *cdc33*, *cdc35*, or *ils1* cells were incubated at the nonpermissive temperature for three to four generation-times as determined at 23°C (Table II). Cells in the arrest caused by sulfur starvation showed a slight increase in the cell volume. Increases in the cell volume observed with *cdc28*, *cdc4*, and *cdc24* mutants at nonpermissive temperature were much more pronounced than those with other three *cdc* mutants and *ils1*. It might be possible, therefore, that the coordinative repression of macromolecule syntheses is the major cause for maintaining the cell volume constant.

Changes in the protein content per cell during the arrest were found proportional to those in the cell volume for each of the G_1 arrests (data not shown).

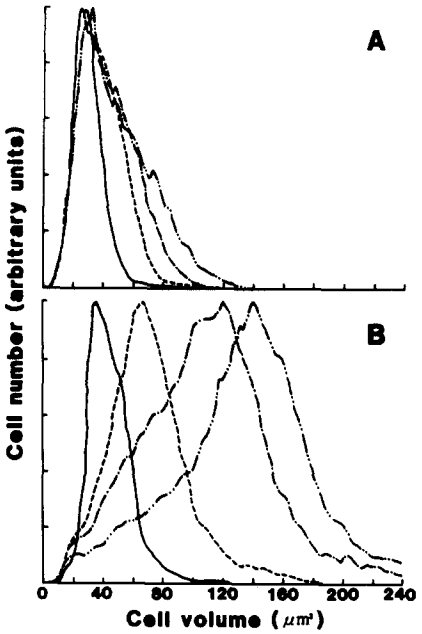

FIGURE 2 Changes in cell volume after shift to nonpermissive temperature. Cells (1.5×10^6/ml) of *cdc25* (*A*) and *cdc28* (*B*) mutants grown at 23°C in SYE medium were transferred to nonpermissive temperature. Volume distribution was determined 0 (——), 3.5 (-----), 6 (-·-·-·-·-) and 8.5 (-·-·-·-) h after the shift.

Stringent Response in G_1 or G_0 Arrest and Its Relaxation by Cycloheximide

The results shown above seem to indicate that cells arrested initially at G_1 entered and went deeper into G_0 when sulfur starvation or either one of *cdc25*, *cdc33*, *cdc35*, and *ils1* mutations was used for the arrest of cell growth. When cells entered G_0, unbalanced growth should be avoided, which might eventually result in cell death otherwise. Therefore, the transition from G_1 to G_0 may involve a stringent response by which the synthesis of proteins and that of RNAs are coordinatively regulated.

We determined the rate of protein synthesis and accumulation of RNA during G_1 arrests caused by various methods. The rate of protein synthesis was found for sulfur-starved cells and the above three *cdc* mutants and *ils1* to decrease by 43–87% within 1 h after the shift to nonpermissive conditions, whereas it remained almost constant or increased for other *cdc* mutants and the α-factor blockade (Fig. 3).

RNA accumulation as measured by incorporation of [^{3}H]uracil was greatly reduced by arrests that were supposed to result in G_0, except for the arrest caused by *cdc25* mutation (Fig. 4, *A–D* and *I*). A relatively low level of the reduction observed with *cdc25* cells may correlate with the fact that it divides more than once after a shift to the nonpermissive temperature (see below). The reduction was significant when *cdc25* cells were incubated at 36°C for 11 h or longer (data not shown). By contrast, RNA accumulation was found to increase for the arrests of *cdc4* or *cdc24* cells at the nonpermissive temperature (Fig. 4, *F* and *G*). The level of the reduction was intermediate for *cdc28* cells (Fig. 4 *E*). RNA accumulation was not affected by α-factor during the initial

TABLE II

Changes in Cell Volume during Incubation under Nonpermissive Conditions

Strain	Mutation	Method for arrest	Incubation time*	Cell Volume‡		Viability§
				μm^3	Ratio‖	
X2180-1A		Sulfur starvation	0	31	1.0	95
			64	39	1.3	94
321	*cdc25-1*	36°C	0	31	1.0	92
			8.5	49	1.6	81
321	*cdc25-1*	α-Factor	0	32	1.0	98
			8.5	84	2.6	94
E17	*cdc33-1*	36°C	0	30	1.0	92
			18	54	1.8	83
BR214-4a	*cdc35*	36°C	0	30	1.0	92
			8.5	38	1.3	68
185-3-4	*cdc28-1*	38°C	0	46	1.0	97
			8.5	135	2.9	72
182-6-3	*cdc24-1*	36°C	0	34	1.0	93
			8.5	197	5.8	65
314	*cdc4-1*	36°C	0	33	1.0	92
			8.5	137	4.2	25
341	*ils1-1*	32°C	0	31	1.0	92
			8.5	56	1.8	83
A364A		–¶	0	35	1.0	96
			8.5	40	1.1	94

*Time after the shift from permissive to nonpermissive conditions.

‡Mean values.

‖Values were normalized by those at time 0.

§Viability was determined by the methylene blue methods (32).

¶Cells were incubated at 36°C as a control.

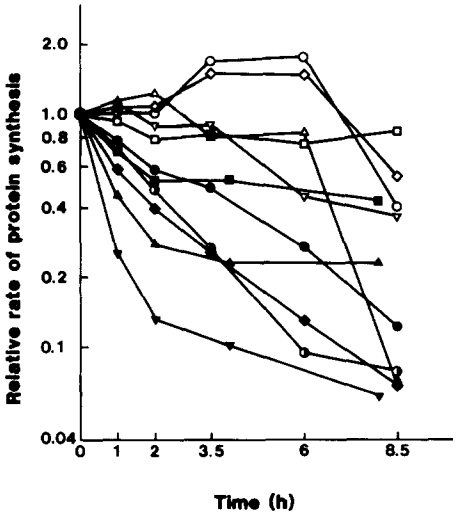

FIGURE 3 Change in protein synthesis after shift to nonpermissive conditions. Exponentially growing cultures of the indicated strains were shifted to nonpermissive conditions. 0.2-ml aliquots of the culture received 4 μCi [^{35}S]methionine (specific activity of 0.3 and 10 Ci/mmol for *cdc* mutants and X2180-1A, respectively; Amersham Japan) or 1 μCi [^{3}H]leucine (146 Ci/mmole; Amersham Japan) and incubated for 10 min. The radioactivity associated with 5% TCA-insoluble materials was determined. Protein content was determined according to the methods described by Stewart (38). The rate of protein synthesis per milligram of protein at various times after the shift was divided by that at time 0. A364A (○), *cdc24* (◇), *cdc25* blocked by α-factor at 23°C (□), *cdc4* (△), *cdc28* (▽), *cdc25* (●), *cdc35* (◆), *cdc33* (■), sulfur starvation with [^{35}S]methionine (▲), sulfur starvation with [^{3}H]leucine (▼), *ils1* (◑).

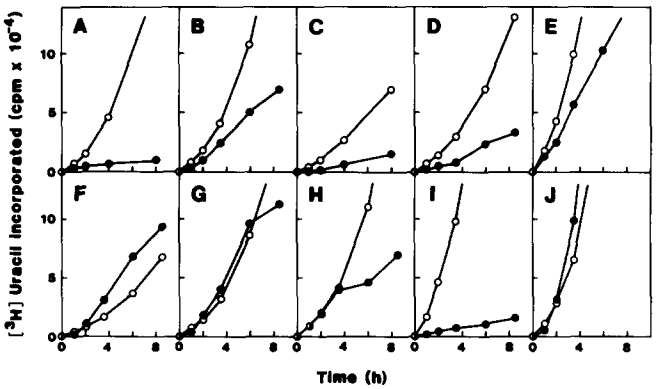

FIGURE 4 Accumulation of synthesized RNA in nonpermissive conditions. Exponentially growing cultures of the indicated strains received 38 μCi/ml of [6-^{3}H]uracil. The specific activity of [6-^{3}H]-uracil was 1.76 and 0.18 Ci/mmol for SYE and SD10-S medium, respectively. Nonradioactive uracil was added to 200 μM in SD10-S. Each of the cultures were divided into two parts; one was incubated further in permissive conditions and the other was shifted to nonpermissive conditions. The radioactivity associated with TCA-insoluble materials in 0.2 ml culture was periodically determined. (*A*) Sulfur starvation; (*B*) *cdc25*; (*C*) *cdc33*; (*D*) *cdc35*; (*E*) *cdc28*; (*F*) *cdc24*; (*G*) *cdc4*; (*H*) α-factor; (*I*) *ils1*; (*J*) a control experiment with A364A. Permissive conditions (○), nonpermissive conditions (●).

3.5 h (Fig. 4*H*) although cell division was completely arrested by α-factor till 2 h after the addition of the factor (data not shown).

The relaxation of the stringent control of rRNA synthesis has been observed in eucaryotic (18, 29) and procaryotic cells

(20) when an inhibitor of protein synthesis was added to these arrested cells. We have tested, therefore, whether or not rRNA synthesis showed a stringent response and, in addition, whether the relaxation of the response by cycloheximide was observed when cells were incubated under the conditions leading to G_0 arrest.

Mutant *cdc33* cells were continuously labeled with [^{14}C]-uracil for several generations at 23°C and were pulsed with [^{3}H]uracil under various conditions; at 23°C, at 23°C in the presence of cycloheximide, at 36°C after incubation for 2 h at 36°C, and at 36°C in the presence of cycloheximide after incubation for 2 h at 36°C. RNA fractions were extracted and analyzed on PAGE. The radioactivity associated with 18S, 25S, and 35S precursor rRNAs was determined (Table III). The results showed that the rate of rRNA synthesis was reduced to 52% 2 h after the shift to 36°C. When cycloheximide was added upon the shift up of the temperature, it was not significantly reduced at 36°C. When the same drug was added to the culture at 23°C, an inhibition by 50% was observed. From these results, a relaxation index was calculated to be 2.3 with *cdc33* cells incubated at 36°C for 2 h.

A relaxation index for the parental strain A364A was 1.0, which indicates that the stringent control mechanism did not suppress the synthesis of rRNA at 36°C. For three other arrests leading to G_0, sulfur starvation, and *cdc25* and *ils1* mutations, indices were obtained to be 3.0, 2.1 and 9.8, respectively (Table III). Indices obtained with *cdc* mutants that were not arrested in G_0 at the nonpermissive temperature were not significantly higher than 1.0. Cells of *cdc35* mutant gave an intermediate index.

Sequence of Steps along the Cell Cycle Sensitive to cdc and ils Mutations, Sulfur Starvation, and α-Factor

By the phenotypic analysis of double mutants and the reciprocal shift experiment, Hartwell and his colleagues have investigated the sequence of steps along the cell cycle that were sensitive to the mating factor of *cdc* mutations (14, 15). They have shown that a step sensitive to α-factor was indistinguishable from a step sensitive to the *cdc28* mutation but was mapped upstream compared to those sensitive to the *cdc4* or *cdc24* mutations.

We have also performed reciprocal shift experiments with combinations of the α-factor blockade and each of three *cdc* mutations, *cdc25*, *cdc33*, and *cdc35* and an *ils1* mutation (Fig. 5). For instance, a culture of *cdc25* cells arrested at 36°C received α-factor just before the shift to 23°C. Reversely, *cdc25* cells blocked at 23°C by α-factor were shifted to 36°C when the blockade by the mating factor was released. The results showed that bud emergence was observed only in the latter shift but not in the former (Fig. 5, *A* and *B*), suggesting that the step sensitive to the *cdc25* mutation might precede the step blocked by α-factor in the cell cycle. Similar results were obtained with *ils1* cells (Fig. 5, *E* and *F*). However, Hartwell et al. (13) pointed out that *cdc25* cells divided more than once at 36°C, which suggested that the product of the mutant *cdc25* gene might not be *ts* but the synthesis or assembly of the gene product might be. Therefore, if the product of the *cdc25* gene could persist for longer than a generation, the results shown in Fig. 5, *A* and *B*, are consistent with the possibility that the step blocked by α-factor might be the same as that sensitive to the *cdc25* mutation at 36°C. By contrast, we have observed that cell division did not take place more than once in the *ils1* mutant after the shift to 32°C (data not shown). This suggests that the *ILS1*-mediated step precedes the α-factor-sensitive step in the cell cycle.

The results obtained with *cdc33* (Figs. 5, *C* and *D*) or *cdc35* (data not shown) mutations and α-factor showed that the steps sensitive to these two *cdc* mutations were not distinguished from the step blocked by α-factor. A map of the sequence of steps blocked by *ts*-mutations or α-factor is shown in Fig. 6.

DISCUSSION

Two Types of G_1 Arrests

In this study, we investigated growth recovery of yeast cells after they had been arrested for different times at various points in G_1 of the cell cycle. To bring about G_1 arrests, we used four different methods; (*a*) starvation for a nutrient, sulfur (11, 14); (*b*) incubation at nonpermissive temperature

TABLE III

Reduction of rRNA Synthesis during G_1 Arrest and Its Relaxation by Cycloheximide

Mutant or treatment	Absolute rate of rRNA synthesis at the permissive conditions*	Relative rate of rRNA synthesis‡				Relaxation Index‖ (D/C)/(B/A)
		A	B	C	D	
		%				
cdc25	19	100	27	77	44	2.1
cdc33	9.2	100	46	52	54	2.3
cdc35	12	100	57	50	42	1.5
cdc28	19	100	22	133	35	1.2
Control (A364A)	18	100	28	181	49	1.0
Sulfur starvation§	21¶	100	54	7.9	13	3.0
ils1	22	100	19	4.2	7.8	9.8

A, permissive conditions. *B*, permissive conditions in the presence of cycloheximide. *C*, nonpermissive conditions. *D*, nonpermissive conditions in the presence of cycloheximide.

* The absolute rate of rRNA synthesis (Σ [^{3}H]dpm/Σ [^{14}C]dpm) was determined as described in Materials and Methods.

‡ The rate obtained with each strain at permissive conditions without cycloheximide was expressed as 100. Based on this, relative rates were determined.

‖ A relaxation index was given by (D/C)/(B/A).

§ A strain X2180-1A was used.

¶ The ratio of the specific activity of [^{3}H]uracil to that of [^{14}C]uracil was three times lower in labeling medium for strain X2180-1A than in that for the other strains (see Materials and Methods). The absolute rate of rRNA synthesis of this strain was expressed as 3 × Σ [^{3}H]dpm/Σ [^{14}C]dpm.

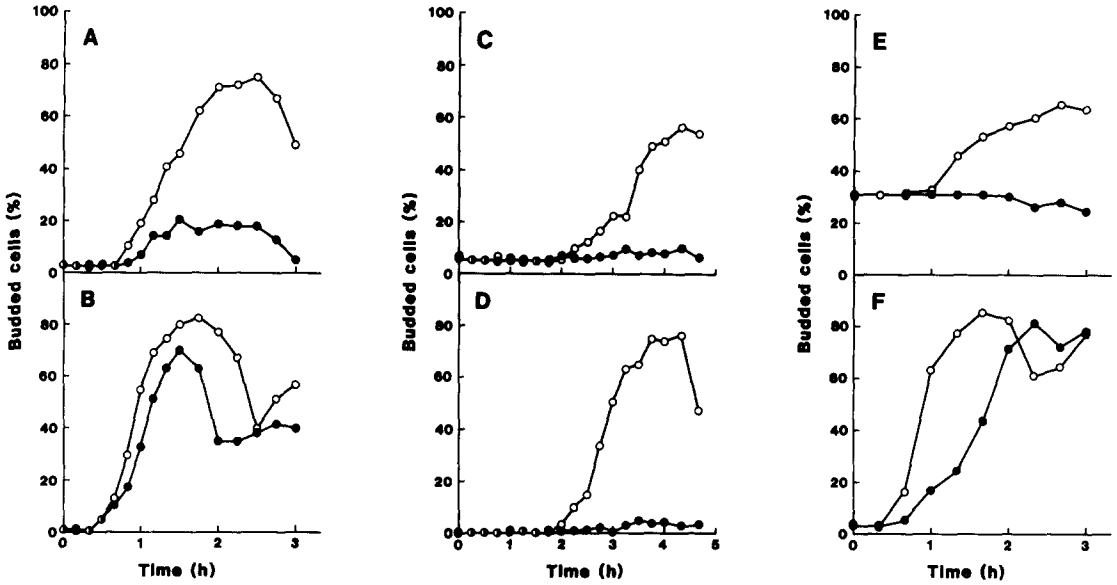

FIGURE 5 Reciprocal shift experiments to determine sequence of steps sensitive to *cdc* and *ils* mutations and α-factor. (*A* and *B*) *cdc25* vs. α-factor block: (*A*) *cdc25* cells arrested at 36°C for 6 h were shifted to 23°C at time 0. 12.5 units/ml (●) or no (○) α-factor was added at time 0. (*B*) *cdc25* cells arrested by 12.5 units/ml α factor for 3.5 h at 23°C were transferred to medium free of α-factor at time 0. Temperature was raised to 36°C (●) or maintained at 23°C (○) at time 0. (*C* and *D*) *cdc33* vs. α-factor block: the experimental procedures were the same as those for *A* and *B* except that *cdc33* cells arrested at 36°C for 13 h were used in *C*. (*E* and *F*) *ils1* vs. α-factor block: the experimental procedures were the same as those for *A* and *B* except temperature block was performed at 32°C. Bud emergence was determined as a function of time after the shift from one restrictive condition to the other.

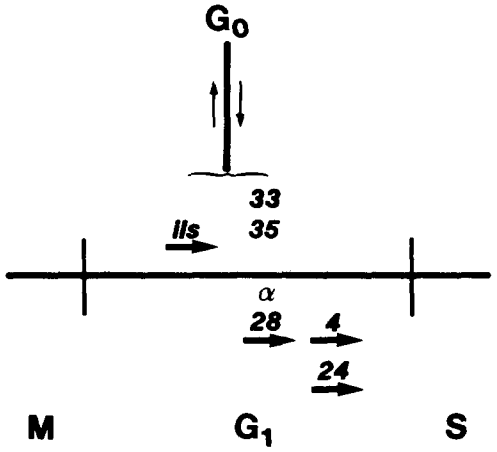

FIGURE 6 Map of arrest points used in this study. Transition from G_1 to G_0 was induced upstream from or at the step sensitive to α factor or *cdc28* mutation. Sensitive steps to *cdc* mutations, *ils1* mutation, and α-factor block were shown by corresponding numbers, *ils* and α, respectively. The step sensitive to sulfur starvation or *cdc25* mutation precedes or is equivalent to that sensitive to α-factor (see reference 14 and the text).

of *cdc* mutants in which cell cycle steps before the onset of DNA synthesis and/or budding are temperature-sensitive (11, 14); (*c*) incubation of haploid cells of *a* mating type with α-factor (2); and (*d*) incubation of cells with *ts*-mutation of aminoacyl-tRNA synthetase at the nonpermissive temperature (44). Based on kinetics of the growth recovery of arrested cells, we classified G_1 arrests into two types.

The first type of G_1 arrest is characterized by causing a subsequent delay of the onset of bud emergence upon the shift to permissive conditions. In addition, the length of the delay depends upon the duration that cells have been arrested. Arrests produced by sulfur starvation, *ts*-mutation of aminoacyl-tRNA synthetase or *cdc25*, *cdc33*, or *cdc35* mutations belong to the first type. The second type of G_1 arrest shows the characteristic that the duration of arrest does not influence kinetics of the restimulation. Arrests produced by *cdc28* or *cdc24* mutations or α-factor block belong to the second type.

The type 1 arrest appears to be similar to the state of cells previously designated as G_0 or the resting state in cultured mammalian and avian cells (1, 19, 25, 40). Using cultured human fibroblasts, Augenlicht and Baserga (1) have found that a lag before the onset of DNA synthesis elongated when cells that had been arrested for longer periods were stimulated. They interpreted their findings as indicating that fibroblasts in a confluent culture first rested in G_1 and went into G_0 as these cells remained arrested for longer periods. In addition, when porcine lymphocytes were stimulated successively twice by pulse exposures to a stimulus, it was found that the second stimulation of DNA synthesis occurred much more rapidly than the first one (23). This suggests that stimulated and recently divided lymphocytes, which are thought to be G_1 cells, responded to the stimulus much more rapidly than G_0 lymphocytes freshly prepared from peripheral blood. We have recently observed that kinetics of the stimulation of G_1 lymphocytes was changed and became similar to that of G_0 lymphocytes as G_1 lymphocytes were incubated in the absence of the stimulus (19). It would be possible to conclude from the results previously obtained with fibroblasts and lymphocytes that the change from the rapidly responding state to the slowly responding state, which is induced by the incubation of these cells under appropriate nonpermissive conditions, is one of the common features associated with eucaryotic cells entering G_0 from G_1. Accordingly, the type 1 arrest of yeast cells appears, but the type 2 does not, to meet this criterion for G_1 arrest leading to G_0.

Reed (31) classified *ts* mutants defective in the start event

of the cell division cycle into two classes; when shifted to the nonpermissive temperature, Class-I mutants continue to grow and retain the capacity to conjugate but Class-II mutants, like starved cells, do not grow and do not conjugate. The two arrest-types that we defined, types 1 and 2 appear to be similar to the arrests caused by Class-II and Class-I mutations (31), respectively, though we dealt with some nonstart mutants, such as *cdc4* and *cdc24*, in addition to start mutants.

Johnston et al. (17) have shown that cells arrested in stationary phase that had been initially smaller in size required more time before the beginning of bud emergence when they were diluted in a fresh medium. It was suggested, in addition, that a specific early event in G_1, at or before the event controlled by the *CDC28* gene product, cannot be completed until a critical cell size is attained (17). Our present results do not necessarily contradict their observations, but show further that the cell volume is not the unique factor determining a lag before the growth recovery because at least the majority of arrested cells by *cdc25*, *cdc33*, *cdc35*, or *ils1* mutation were larger than growing cells at permissive temperature (Table II). If transition to G_0 is directed by, as we suppose, the mechanism that coordinatively depresses macromolecule synthesis, a striking increase in cell volume would not be expected. This was met by the result shown in Table II.

Variable division delay was produced by radiation (36) or by inhibitor of protein synthesis (42) in a dose- or exposure time-dependent manner. This appears to be similar to variable delay in the initiation of budding or DNA synthesis that we herein dealt with. However, it has been shown that the division delay was determined at point(s) in G_2 (for review, see reference 27) and, therefore, was irrelevant to specific G_1 arrests leading to G_0.

It should be noted that we used the mode of growth recovery from the restrictive conditions to discriminate between G_0 and G_1 arrest. Therefore, molecular basis of the G_0 arrest remains to be unraveled.

Induction of G_0 Transition at Specific Point(s) in G_1

Based upon the results previously reported by others (14, 15) and on our results, arrest points in the G_1 interval are mapped in Fig. 6. As mentioned above, the reciprocal shift experiments showed (*a*) an arrest point by *ils1* mutation precedes the arrest point by α-factor (Fig. 5), (*b*) an arrest point by *cdc25* mutation (Fig. 5) or sulfur starvation (14) precedes or is equivalent to that by α-factor, and (*c*) an arrest point by *cdc33* or *cdc35* is not distinguishable from that by α-factor (Fig. 5). However, arrested cells produced by nutrient starvation, or *cdc25*, *cdc33*, or *cdc35* mutations were shown to have spindle-pole-bodies bearing no satellites whereas those arrested by α-factor or *cdc28* mutation had spindle-pole-bodies that bear satellites but did not enlarge appreciably (4). We suggest, therefore, that G_0 transition might be induced when yeast cells arrested at point(s) in G_1 earlier than the point sensitive to α-factor.

Our results obtained in the present study are compatible with the concept of the restriction point (R-point) proposed by Pardee (28). He has demonstrated that animal cells are put into the same quiescent state by different methods such as serum depletion or high cell density and that arrested cells re-enter the cell cycle through a unique R-point in G_1 when the restriction for growth is released. We have shown that mutations in at least three different *CDC* genes and a mutation in isoleucyl-tRNA synthetase whose expression was required in early stages of the cell cycle led yeast cells to the specific growth arrest, G_0. These arrested cells had not passed the critical point(s) for growth mapped upstream from or closely to the α-factor-sensitive point.

A point along the cell cycle defined as the "start" point by Hartwell and his associates (11, 14) has been shown to be critical for the control of cell proliferation in the yeast. Shilo et al. (33, 34) showed that reinitiation of the cell cycle follows first-order kinetics in cells arrested before the "start" point but not in those arrested after the "start" point. The former type of arrest was induced by *cdc25*, *cdc35*, *cdc28*, or *tra3* (46) mutations, α-factor or stationary phase and the latter type was caused by *cdc24* mutation. When our results shown in Fig. 1 were replotted according to the plotting method by Shilo et al. (33, 34), they revealed that reinitiation of cell proliferation after arrests leading to G_0 or by α-factor follows first-order kinetics (data not shown). Furthermore, duration of the arrest leading to G_0 affected the lag before the onset of budding without altering the rate constant of the first-order reaction. By contrast, the duration of the arrest by α-factor that is assumed not to lead to G_0 did not affect the lag or the rate.

Mechanism of G_0 Transition

Our results showed that G_0 arrest of yeast cells that resulted from various treatments was always preceded by reduction in the synthesis of both protein and RNA. The regulatory mechanism directing such a coordinative depression of macromolecule synthesis seems to resemble the stringent control system in bacterial microorganisms (5, 8). This view was supported by the finding that cycloheximide relaxed the inhibition of rRNA synthesis caused by the above treatments except *cdc35*. It has been previously shown that the stringent response of RNA synthesis was induced when yeast cells were starved for an essential amino acid, tyrosine (18, 45), though the mediator of the response has not been identified (35, 45).

In addition to the phenotypic similarity to the stringent response in bacteria, the mechanism operating in the transition to G_0 in eucaryotic cells appears to have an intriguing feature. Inasmuch as transition to G_0 in the yeast was induced by a defect in either one of, at least, three different *CDC* genes, *CDC25*, *CDC33* and *CDC35*, or *ILS1* gene, yeast cells must be endowed with an ability to respond different cellular signals and bring about the same state, G_0.

Finally, we suggest that yeast cells, especially strains with certain *cdc* mutants, could provide a useful system in which biochemical events associated with G_0 transition and those events that continue during a simple G_1 arrest could be discriminatingly investigated. Using the yeast system, we have recently found that high molecular weight species of heat shock proteins were durably synthesized in G_0 (manuscript submitted for publication).

We thank Drs. Y. Anraku and Y. Ohsumi (University of Tokyo) for facilities for the preparation of α-factor. We also thank Drs. L. H. Hartwell (University of Washington) and Y. Ohsumi and the Yeast Genetic Stock Center (University of California) for yeast strains.

This work was supported in part by grants-in-aid from the Ministry of Education, Science and Culture of Japan.

Received for publication 8 April 1983, and in revised form 12 December 1983.

REFERENCES

1. Augenlicht, L. H., and R. Baserga. 1974. Changes in the G_0 state of WI-38 fibroblasts at different times after confluence. *Exp. Cell Res.* 89:255–262.
2. Bücking-Throm, E., W. Duntz, L. H. Hartwell, and T. N. Manney. 1973. Reversible arrest of haploid yeast cells at the initiation of DNA synthesis by a diffusible sex factor. *Exp. Cell Res.* 76:99–110.
3. Burstin, S. J., H. K. Meiss, and C. Basilico. 1974. A temperature-sensitive cell cycle mutant of the BHK cell line. *J. Cell. Physiol.* 84:397–408.
4. Byers, B. 1981. Multiple roles of the spindle pole bodies in the life cycle of *Saccharomyces cerevisiae*. *In* Molecular Genetics in Yeast. Alfred Benzen Symposium. 19. D. von Wettsetin, A. Stenberup, M. Kielland-Drandt, and J. Friis. editors. Munksgaard, Copenhagen. pp. 119–133.
5. Cashel, M. 1975. Regulation of bacterial ppGpp and pppGpp. *Annu. Rev. Microbiol.* 29:301–318.
6. Duntze, W., D. Stötzler, E. Bücking-Throm, and S. Kalbitzer. 1973. Purification and partial characterization of α-factor, a mating-type specific inhibitor of cell reproduction from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 35:357–365.
7. Fahey, J. L. 1974. Control of proliferation in the immune system. *In* Control of Proliferation of Animal Cells. *Cold Spring Harbor Conf. Cell Proliferation.* 11:379–392.
8. Gallant, J. A. 1979. Stringent control in *E. coli*. *Annu. Rev. Genet.* 13:393–415.
9. Hartwell, L. H. 1970. Periodic density fluctuation during the yeast cell cycle and the selection of synchronous cultures. *J. Bacteriol.* 104:1280–1285.
10. Hartwell, L. H. 1973. Three additional genes required for deoxyribonucleic acid synthesis in *Saccharomyces cerevisiae*. *J. Bacteriol.* 115:966–974.
11. Hartwell, L. H. 1974. *Saccharomyces cerevisiae* cell cycle. *Bacteriol. Rev.* 38:164–198.
12. Hartwell, L. H., and C. S. McLaughlin. 1968. Mutants of yeast with temperature-sensitive isoleucyl-tRNA synthetases. *Proc. Natl. Acad. Sci. USA.* 59:422–428.
13. Hartwell, L. H., R. K. Mortimer, J. Culotti, and M. Colotti. 1973. Genetic control of the cell division cycle in yeast. V. Genetic analysis of *cdc* mutants. *Genetics.* 74:267–286.
14. Hartwell, L. H., J. Culotti, J. R. Pringle, and B. J. Reid. 1974. Genetic control of the cell division cycle in yeast. *Science (Wash. DC).* 183:46–51.
15. Hereford, L. M., and L. H. Hartwell. 1974. Sequential gene function in the initiation of *Saccharomyces cerevisiae* DNA synthesis. *J. Mol. Biol.* 84:445–461.
16. Jarvik, J., and D. Botstein. 1973. A genetic method for determining the order of events in a biological pathway. *Proc. Natl. Acad. Sci. USA.* 70:2046–2050.
17. Johnston, G. C., J. R. Pringle, and L. H. Hartwell. 1977. Coordination of growth with cell division in the yeast *Saccharomyces cerevisiae*. *Exp. Cell Res.* 105:79–98.
18. Kelker, H. C., and A. O. Pogo. 1980. The stringent and relaxed phenomena in *Saccharomyces cerevisiae*. *J. Biol. Chem.* 255:1526–1535.
19. Kumagai, J., H. Akiyama, S. Iwashita, H. Iida, and I. Yahara. 1981. *In vitro* regeneration of resting lymphocytes from stimulated lymphocytes and its inhibition by insulin. *J. Immunol.* 126:1249–1254.
20. Kurland, C. G., and O. Maaløe. 1962. Regulation of ribosomal and transfer RNA synthesis. *J. Mol. Biol.* 4:193–210.
21. Lajtha, L. G., B. I. Lord, T. M. Dexter, E. G. Wright, and T. D. Allen. 1978. Interrelationship of differentiation and proliferation control of hematopoietic stem cells. *In* Cell Differentiation and Neoplasia. G. F. Saunders, editor. Raven Press, New York. pp. 179–193.
22. Ling, N. R., and P. J. L. Holt. 1967. The activation and reactivation of peripheral lymphocytes in culture. *J. Cell Sci.* 2:57–70.
23. Ling, N. R., and J. E. Key. 1975. Lymphocytes Stimulation. American Elsevier Publishing Co., Inc., New York. 365–366.
24. Loening, U. E. 1969. The determination of the molecular weight of ribonucleic acid by polyacrylamide-gel electrophoresis: the effects of changes in conformation. *Biochem. J.* 113:131–138.
25. Martin, R. G., and S. Stein. 1976. Resting state in normal and simian virus 40 transformed Chinese hamster lung cells. *Proc. Natl. Acad. Sci. USA.* 73:1655–1659.
26. McLaughlin, C. S., P. T. Magee, and L. H. Hartwell. 1969. Role of isoleucyl-transfer ribonucleic acid synthetase in ribonucleic acid synthesis and enzyme repression in yeast. *J. Bacteriol.* 100:579–584.
27. Mitchison, J. M. 1971. The Biology of the Cell Cycle. Cambridge University Press, Cambridge. pp. 221–244.
28. Pardee, A. B. 1974. A restriction point for control of normal animal cell proliferation. *Proc. Natl. Acad. Sci. USA* 71:1286–1290.
29. Pogo, A. O., and V. J. Zbrzezna. 1978. Cycloheximide stimulates ribosomal RNA transcription in amino acid-starved ascites tumor cells. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 88:135–138.
30. Pringle, J. R., and L. H. Hartwell. 1981. The *Saccharomyces cerevisiae* cell cycle. *In* The Molecular Biology of the Yeast sccharomyces: Life Cycle and Inheritance. J. N. Strathern, E. W. Jones, and J. R. Broach, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. pp. 97–142.
31. Reed, S. I. 1980. The selection of *S. cerevisiae* mutants defective in the start event of cell division. *Genetics.* 95:561–577.
32. Rose, A. H. 1975. Growth and handling of yeasts. *Methods Cell Biol.* XII:1–16.
33. Shilo, B., V. Shilo, and G. Simchen. 1976. Cell-cycle initiation in yeast follows first-order kinetics. *Nature (Lond.).* 264:767–770.
34. Shilo, B., V. Shilo, and G. Simchen. 1977. Transition probability and cell-cycle initiation in yeast. *Nature (Lond.).* 267:648–649.
35. Silverman, R. H., and A. G. Atherly. 1979. The search for guanosine tetraphosphate (ppGpp) and other unusual nucleotides in eucaryotes. *Microbiol. Rev.* 43:27–41.
36. Sinclair, W. K. 1968. Cyclic x-ray response in mammalian cells *in vitro*. *Radiat. Res.* 33:620–643.
37. Slater, M. L., S. O. Sharrow, and J. J. Gart. 1977. Cell cycle of *Saccharomyces cerevisiae* in populations growing at different rates. *Proc. Natl. Acad. Sci. USA.* 74:3850–3854.
38. Stewart, P. R. 1975. Analytical methods for yeasts. *Methods Cell Biol.* XII:111–147.
39. Stayner, L., and M. R. Schwarz. 1969. The response of long- and short-lived small lymphocytes of the rat to pokeweed mitogen. *J. Immunol.* 102:1260–1267.
40. Temin, H. M. 1971. Stimulation by serum of multiplication of stationary chicken cells. *J. Cell. Physiol.* 78:161–170.
41. Tobey, R. A. 1973. Production and characterization of mammalian cells reversibly arrested in G_1 by growth in isoleucine-deficient medium. *Methods Cell Biol.* VI:67–112.
42. Tobey, R. A., E. C. Anderson, and D. F. Petersen. 1966. RNA stability and protein synthesis in relation to the division of mammalian cells. *Proc. Natl. Acad. Sci. USA.* 56:1520–1527.
43. Udem, S. A., and J. R. Warner. 1972. Ribosomal RNA synthesis in *Saccharomyces cerevisiae*. *J. Mol. Biol.* 65:227–242.
44. Unger, M. W., and L. H. Hartwell. 1976. Control of cell division in *Saccharomyces serevisiae* by methionyl-tRNA. *Proc. Natl. Acad. Sci. USA.* 73:1664–1668.
45. Warner, J. R., and C. Gorenstein. 1978. Yeast has a true stringent response. *Nature (Lond.)* 275:338–339.
46. Wolfner, M., D. Yep, F. Messenguy, and G. R. Fink. 1975. Integration of amino acid biosynthesis into the cell cycle of *Saccharomyces cerevisiae*. *J. Mol. Biol.* 96:273–290.